REMARKS

Please refer to the Marked-Up Versions of Specification Pages 5, 6, 11, 13, 14, 19, and 20 attached herewith

Applicants respectfully request that the preliminary amendment described herein be entered into the record prior to calculation of the filing fee and prior to examination and consideration of the above-identified application.

If a telephone conference would be helpful in resolving any issues concerning this communication, please contact Applicants' primary attorney-of record, Douglas P. Mueller (Reg. No. 30,300), at (612) 371.5237.

Respectfully submitted,

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DPM/rw

According to a preferred embodiment according to this method the searching and identifying is conducted by a custom search algorithm, preferably the algorithm is written in Visual Basic and looks for the triplet peak pattern of $(m/z)_1$, $(m/z)_2$) = $(m/z)_1$ – 18.0, and $(m/z)_3 = (m/z)_2 - 28.0$ as well as the doublet pattern of $(m/z)_2$ and (m/z)3, all to within ± 0.5 *m/z* unit. More preferably the product ion spectra of the $[M + Ag]^+$ ion are collected under E_{cm}s, of 1.5, 2.0, 2.5 and 3.0 eV. Preferably the mass spectrometer used according to the method of the invention is a triple quadrupole mass spectrometer or two triple quadrupole mass spectrometers, however, any mass spectrometer capable of tandem mass spectrometry, such as a quadrupole/time-of-flight mass spectrometer, an ion-trap mass spectrometer, or a time-of-flight mass spectrometer amenable to post-source decay or collision-induced dissociation, may be used.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows product ion spectra of the $[M + {}^{107}Ag]^+$ ion of leucine enkephalin, YGGFL, at different E_{cm}s: (a) 1.5 eV, (b) 2.0 eV, (c) 2.5 eV, and (d) composite of (a) to (c). $b^* = [b_n + OH + Ag]^+$, $b = [b_n - H + Ag]^+$, and $a = [a_n - H + Ag]^+$. (SEQ. ID. NO. 1)

Figure 2 shows a product-ion spectrum of the $[M + {}^{107}Ag]^+$ ion of leucine enkephalin, YGGFL, at a linear E_{cm} function from 2.5 eV for *m/z* = 30 to 1.5 eV for *m/z* = 663. (SEQ. ID. NO. 1)

Figure 3 shows a product-ion spectrum of the $[M + {}^{107}Ag]^+$ ion of GGEGG at an E_{cm} of 2.0 eV. (SEQ. ID. NO. 2)

Figure 4 shows the composite product-ion spectrum of the $[M + {}^{109}Ag]^+$ ion of bradykinin, RPPGFSPFR, at E_{cm}s of 2.0 and 2.5 eV. (SEQ. ID. NO. 3)

Figure 5 shows a product-ion spectrum of the $[M + {}^{107}Ag]^+$ ion of dynorphin A fragment 1-7, YGGFLRR, at E_{cm} = 1.9 eV. (SEQ ID NO: 4)

Figure 6 shows the product-ion spectra of tryptic peptides at linear E_{cm} functions (a) from 4.6 to 1.8 eV and (b) from 4.0 to 1.6 eV.

Figure 7 shows a custom search algorithm of the invention written in Visual Basic.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the present inventors have found that they are able to determine the sequences of peptides or proteins by analysing the peptides or proteins in argentinated form using mass spectrometry. In its broad aspect the invention provides a method of analyzing argentinated peptides or proteins using mass spectrometry comprising:

(a) combining an oligopeptide with silver to provide a sample comprising argentiated oligopeptide;

(b) submitting the sample to a mass spectrometer;

(c) performing; scans of silver containing peaks in optimum collision energies;

(d) identifying any doublet or triplet peak pattern;

(e) confirming with Y ions;

(f) determining partial sequence by the mass separation between two successive doublet or triplet patterns.

Mass Spectrometry

Although the data presented in the examples described herein were conducted on two triple quadrupole mass spectrometers on two triple quadrupole mass spectrometers, any mass spectrometers that are capable of tandem mass spectrometry, such as a quadrupole/time-of-flight mass spectrometer, an ion-trap mass spectrometer, or a time-of-flight mass spectrometer amenable to post-source decay or collision-induced dissociation, may also be used, with appropriate adjustments known to those skilled in the art. The ions may be generated by a number of methods including electrospray, MALDI (matrix-assisted laser desorption ionization) and FAB (fast-atom bombardment). The method of the invention can be applied to oligopeptides of any length, preferably the number of amino acid residues is between 3 and 20.

Where prepared oligopeptides of unknown sequence are to be

characteristics of the triplet pattern of product-ion peaks.

EXAMPLE 1

Figure 1 shows that the product-ion spectra of the $[M + {}^{107}Ag]^+$ ion (silver has two stable isotopes, ^{107}Ag and ^{109}Ag, of almost equal abundance; the product-ion spectra shown in this article will be those of either one of the two isotopes) of leucine enkephalin, YGGFL, collected under different E_{cm}s: (a) 1.5, (b) 2.0, and (c) 2.5 eV; (d) is the composite of (a), (b), and (c). It is well-known that individual product-ion yield is strongly dependent on collision energy (Dawson et al. (*Org. Mass Spectrom.* 17, 205-211 (1982); Dawson et ala. (*Org. Mass Spectrom.* 212-217 (1982)); a necessary step in sequencing is to acquire product-ion spectra under several collision energies to find the best spectra for sequencing. Summing the spectra to produce a composite provides a convenient way of presenting a minimal number of searchable mass spectra to the triplet/doublet identification algorithm. An alternative method to generate a wide range of searchable product ions is to acquire a product-ion spectrum with *m/z*-dependent E_{cm} function. Figure 2 shows such a product-ion spectrum also for the $[M + {}^{107}Ag]^+$ ion of leucine enkephalin. It was acquired with a linear E_{cm} function from 2.5 eV for *m/z* = 30 to 1.5 eV for *m/z* = 663, but otherwise under the same experimental conditions as those in Figure 1 (the scan time equalled the total scan time of the three individual E_{cm}s). It is readily apparent that the spectra are not identical (due to differences in exact collision energies), but the overall spectral quality of the two is similar.

Figures 1d and 2 are of such quality that the amino acid sequence of leucine enkephalin can be read directly. The search algorithm looks for a triplet pattern of peaks, whose members are 18 and 28 *m/z* units apart, and assigns these as $[b_n + OH + Ag]^+$, $[b_n - H + Ag]^+$, and $[a_n - H + Ag]^+$, in decreasing *m/z* values (the product ions are labeled as b*, b, and a, respectively, in the figures). The amino acid residues that are cleaved from the C terminus can be determined from the differences in the *m/z* values of the $[M + Ag]^+$ ion and the appropriate triplet members. For example, in Figure 2 the first triplet that may be identified starting from *m/z* 662.2 is *m/z* 548.9, 531.2, and 503.1 (differences of *m/z* values, $\Delta m/z$ = 17.7 and 28.1). The peak with the largest *m/z* value in the triplet is the $[b_n + OH + Ag]^+$ ion,

ion of tyrosine (theoretical $\Delta m/z$ = 136.2). Thus, the determined amino acid sequence of leucine enkephalin is Y-G-G-F-L/I, which is correct. <-(SEQ.ID.NO.1/SEQ.ID.NO.5)--

EXAMPLE 2

Figure 3 shows a product-ion spectrum of the $[M + {}^{107}Ag]^+$ ion of GGEGG, glycylglycylglutamylglycylglycine. Sequencing of the argentinated <-(SEQ.ID.NO.2)-- peptide is straightforward and similar to that discussed in Example 1 for leucine enkephalin. The difference in m/z values between the $[M + Ag]^+$ ion and the first $[b_n + OH + Ag]^+$ ion is 482.1 - 425.1 = 57.0, which identifies the C-terminal residue as glycine (theoretical $\Delta m/z$ = 57.0). The difference in m/z values between the first $[b_n + OH + Ag]^+$ and the second $[b_n + OH + Ag]^+$ is 425.1-368.0 = 57.1; this identifies the residue preceding the C-terminus as, again, glycine. Repeating the procedure yields the next residue, glutamic acid (experimental $\Delta m/z$ = 128.9 versus theoretical $\Delta m/z$ = 129.0). The triplet peak pattern disappears beyond glutamic acid; however, manual interpretation reveals further sequence information. The $[a_1 - H + Ag]^+$ in this product-ion spectrum is weak. However, the small m/z value of the $[a_n - H + Ag]^+$ ion of the last triplet, 193.1, strongly suggests that it is the $[a_2 - H + Ag]^+$ ion. This makes G-G as the only possible option for the N-terminal and second residues (theoretical m/z = 193.0). Furthermore, Figure 3 shows a small peak at m/z = 136.0, which is assignable as the $[a_1 - H + Ag]^+$ ion (theoretical m/z = 136.0, assuming the first residue is glycine). Thus, the determined sequence is G-G-E-G-G <-(SEQ.ID.NO.2)--

EXAMPLE 3

The following example relates to a longer peptide bradykinin. A product-ion spectrum of the $[M + {}^{109}Ag]^+$ ion of bradykinin, RPPGFSPFR, is <-(SEQ.ID.NO.3)-- shown in Figure 4. Sequencing of the $[M + H]^+$ ion of bradykinin is considered difficult because the external proton is believed to be sequestered by the highly basic guanidine groups on the side chains of the two arginine residues, thus rendering it unavailable for binding to the amidic functional groups and inducing charge-proximal fragmentation along the peptide backbone (Alexander et al. (1990); Tang et al. (1993); Burlet et al. (1992); Cox et al. (1996); Summerfield et al. (1997)). The Ag + ion, however, appears to bind to many different sites on the peptide, as evident from the relative richness of the fragmentation pattern in Figure 4. Table 1

summarizes the triplets found and the residues identified using the above approach. It is apparent that only a partial sequence of five residues starting from the C-terminal end of the peptide has been solved - FSPFR; the major advantage of sequencing argentinated peptides relative to protonated peptides is the triplet relationship which greatly facilitates product-ion assignment. Bradykinin has the highly basic arginine as its C-terminal residue, which also binds strongly to the silver ion (Lee at al. *J. Am. Soc. Mass Spectrom.* (1998)). In fact, it is our observation that peptides that have C-terminal methionine, lysine, and arginine residues tend to yield relatively strong $[y_n + H + Ag]^+$ product ions (Li et al. (1997); Chu et al. (not published)). In the search algorithm, presence of the corresponding $[y_n + H + Ag]^+$ ion is used as confirmation of the cleaved residue. For bradykinin, the $[y_n + H + Ag]^+$ ions for n = 3-5 have been observed (Figure 4 and Table 1), which confirms results of the triplet search. In Figure 4, the triplet signal corresponding to cleavage of the proline residue is weak; this is actually a confirmation of the proline-residue assignment. Proline is the only residue from which an oxazolone $[b_n - H + Ag]^+$ cannot be formed and of which the relatively weak $[b_n - H + Ag]^+$ ion is believed to be a ketene (Lee et al., *J. Am. Chem. Soc.* (1998)). The assignment of proline can often be confirmed by the presence of the appropriate $[y_n + H + Ag]^+$ ions or the identification of the next triplet; the difference in the *m/z* values of the $[b_n + OH + Ag]^+$ ions for n = 1 and n = 3 is 1010.3 - 766.5 = 243.8, which can only mean a combination of cleavage of phenylalanine and proline.

EXAMPLE 4

Figure 5 shows a product-ion spectrum of the $[M + {}^{107}Ag]^+$ ion of dynorphin A fragment 1-7, YGGFLRR, another highly basic peptide. The triplets and the residues identified using the search algorithm are tabulated in Table 2. Again, the partial sequence determined, - -FLRR, is confirmed by the presence of the appropriate $[y_n + H + Ag]^+$ ions.

EXAMPLE 5

A frequent and valid criticism of the above type of examples is that it is relatively easy to sequence a peptide whose identity is known a *priori*. One of us (IKC) was presented with tryptic digests of proteins unknown to him. He was asked to sequence a number of the tryptic peptides from

Table 3

Sequencing of Tryptic Peptides

peptide	determined sequence	actual sequence
1	-FAGK- (SEQ. ID. NO. 6) --	LIFAGK- (SEQ. ID. NO. 7) --
2	-(L/I)FVK- (SEQ. ID. NO. 8) --	MQIFVK- (SEQ. ID. NO. 9) --
3	-TGK- (SEQ. ID. NO. 10) --	TLTGK- (SEQ. ID. NO. 11) --
4	-DVEK- (SEQ. ID. NO. 12) --	GDVEK- (SEQ. ID. NO. 13) --
5	-V(Q/K)K[a]- (SEQ. ID. NO. 14) --	IFVQK- (SEQ. ID. NO. 15) --

[a] K and Q are isobaric; however, since trypsin cleaves only on the C-terminal side of K and R, the identity of the C-terminal residue is unambiguous, but that of preceding residues is not.

Table 4

Sequence Tag Analyses of Tryptic Peptides
(Protein Molecule Mass: 8565 Da)

(a)	Peptide mass: 647.2 Da (756.1-108.9)	Protein	Mr
	Sequence tag: 227.3/FAG/502.2	swiss|P02248	8564.87
	(335.2 + 1 - 108.9 = 227.3;	spit|Q17001	8563.89
(b)	610.1 + 1 - 108.9 = 502.2)	spit|Q41405	8562.83
	Peptide mass: 764.4 Da (873.3 - 108.9)	t|M21581	8566.91
	Sequence tag: 260.1/L (or I) FV/619.1	t|M22218	8566.91
	(368.0 + 1 - 108.9 = 260.1;		
	727.0 + 1 - 108.9 = 619.1)		

Swiss|P02248, bold sections correspond to the tryptic peptides entered for the search and identification

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLE
DGRTLSDYNIQKESTLHLVLRLRGG -(SEQ.ID.NO.16)--